# High Serum Androstenedione Levels Correlate With Impaired Memory In The Surgically Menopausal Rat: A Replication And New Findings

by

Bryan Walter Camp

A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Arts

Approved December 2011 by the
Graduate Supervisory Committee:

Heather Bimonte-Nelson, Chair
Michael Foster Olive
Cheryl Conrad

ARIZONA STATE UNIVERSITY

December 2012

ABSTRACT

After natural menopause in women, androstenedione becomes the primary hormone secreted by the residual follicle deplete ovaries. Two independent studies, in rodents that had undergone ovarian follicular depletion, found that higher serum androstenedione levels correlated with increased working memory errors. This led to the hypothesis that androstenedione impairs memory. The current study directly tested this hypothesis, examining the cognitive effects of androstenedione administration in a rodent model. Middle-aged ovariectomized rats received vehicle or one of two doses of androstenedione (4 or 8 mg/kg daily). Rats were tested on a spatial working and reference memory maze battery including the water radial arm maze, Morris maze, and delay-match-to-sample task. Results showed that androstenedione at the highest dose impaired reference memory and working memory, including ability to maintain performance as memory demand was elevated. The latter was true for both high temporal demand memory retention of one item of spatial information, as well as the ability to handle multiple items of spatial working memory information. Glutamic acid decarboxylase (GAD) levels were measured in multiple brain regions to determine whether the gamma-aminobutyric acid (GABA) system mediates androstenedione's cognitive impairments. Results showed that higher entorhinal cortex GAD levels were correlated with poorer Morris maze performance, regardless of androstenedione treatment. These findings suggest that

androstenedione, the main hormone produced by the follicle deplete ovary, is detrimental to spatial learning, reference memory, and working memory, and that spatial reference memory performance might be related to the GABAergic system.

ACKNOWLEDGEMENTS

This research was funded by a grant awarded to HAB-N from the National Institute of Aging (AG028084). We thank Thu Huynh for aiding training on the elevated plus maze procedures. We are grateful to Dr. Laurence M. Demers and the Core Endrocrinology Laboratory (Pennsylvania State Hershey Medical Center, Hershey, PA) for performing the hormone assays. We wish to express our sincerest appreciation to Cynthia Zay, Joshua Talboom, Elizabeth Engler-Chiurazzi, and Sarah Mennenga for excellent technical assistance.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

Figure Page

LIST OF TABLES

## LIST OF ABBREVIATIONS

1. Delayed-Match-to-Sample Three Choice Task (DMS)
2. Elevated Plus Maze (EPM)
3. Gamma-Aminobutyric Acid (GABA)
4. Glutamic Acid Decarboxylase (GAD)
5. Inter Trial Interval (ITI)
6. Morris Water Maze (MM)
7. Ovariectomy (OVX)
8. Polyethylene Glycol (PEG)
9. Receptor (R)
10. Reference Memory (RM)
11. 4-Vinylcyclohexene-Diepoxide (VCD)
12. Working Memory Correct (WMC)
13. Working Memory Incorrect (WMI)
14. Water Radial-Arm Maze (RM)

# Introduction

Clinical evidence suggests that cognitive outcomes following ovarian hormone loss differ depending upon whether menopause is surgical or transitional. Research has shown that surgically menopausal women exhibited lower memory scores relative to naturally menopausal women, and age of oophorectomy and greater years since surgery correlated with poorer performance (Nappi *et al.*, 1999; Farrag *et al.*, 2002). Researching menopause in the rodent model has been difficult due to the differences in the mechanisms responsible for the age-related reproductive senescence and the hormonal milieu that follows (Meites & Lu, 1994; Timaras *et al.*, 1995).

In the last decade, a rodent model of menopause has been developed. It has been shown that the industrial chemical 4-vinylcyclohexene diepoxide (VCD) produces follicular depletion in rodents (Mayer et al., 2002). VCD selectively destroys primordial and primary follicles, resulting in follicular depletion and ovarian failure (Mayer *et al.*, 2004). This results in a follicle deplete post-menopausal ovary, and a hormone milieu similar to that seen in naturally menopausal women (Timaras *et al.*, 1995) . The interstitial ovarian tissue yields decreased progesterone and androstenedione, and because estrogens become deplete, this creates in an androgen rich milieu (Mayer et al., 2004). Using this VCD model, in middle-aged female rats, we recently showed that retention of the follicle deplete ovary after transitional menopause

negatively impacts memory (Acosta *et al.*, 2009a). Specifically, removal of residual ovaries after transitional follicular depletion improved cognition relative to retention of residual ovaries (Acosta *et al.*, 2009a), as rats that underwent VCD-induced transitional menopause followed by ovariectomy (OVX) showed better cognitive performance compared to VCD menopausal rats that remained ovary-intact. The only difference between these groups was presence of follicle deplete ovaries. Circulating androstenedione levels related to cognitive detriment, with more working memory errors correlating with higher serum androstenedione levels. These findings are especially noteworthy given that androstenedione is the main hormone released by the post-menopausal ovary, and becomes the main source of plasma estrogens after menopause due to aromatization (Grodin *et al.*, 1973).

If androstenedione can in fact impair memory, as our two prior correlations between cognitive performance and circulating androstenedione levels indicate (Acosta *et al.*, 2009b; Acosta *et al.*, 2010), administering androstenedione at doses that correspond to higher physiological levels to the OVX rat should impair cognitive performance. Here, we directly tested the hypothesis that such androstenedione administration impairs spatial working and reference memory. Two doses of exogenous androstenedione were administered to middle-aged surgically menopausal (OVX) rats, with doses based on previous studies (Lea & Flanagan, 1998; Sprando et al., 2004) showing physiological levels

of androstenedione comparable to those correlating with poor cognitive performance in Acosta et al. (2009a). We tested androstenedione's impact on cognition using a battery of tasks tapping into several memory domains including, the water radial-arm maze (WRAM) which assessed working and reference memory and incorporated memory load increase across trials, the Morris water maze (MM) which tested spatial reference memory, the delayed-match-to-sample three choice task (DMS) which examined working memory, and the elevated plus maze (EPM) which assessed anxiety-like behavior. The WRAM and DMS included retention delays spanning hours, to evaluate high demand memory retention. Additionally, androstenedione was measured and correlated with memory measures, as done previously (Acosta *et al.*, 2009a).

We also sought to evaluate whether the GABAergic system plays a role in the relationship between androstenedione and memory. Research suggests the $GABA_A$ receptor ($GABA_A$-R) modulates cognition. $GABA_A$-R antagonists enhances memory, and $GABA_A$ agonists impair memory (Yonkov & Georgiev, 1985; Yonkov *et al.*, 1987; Brioni *et al.*, 1989; Castellano & McGaugh, 1990; Castellano *et al.*, 1993; Zarrindast *et al.*, 2004). Further, several androgens have been shown to have effects on $GABA_A$ receptors. For example, dehydroepiandrosterone can act as a $GABA_A$R negative allosteric modulator (Birzniece *et al.*, 2006), and has been shown to improve memory in middle-aged and aged mice (Flood & Roberts, 1988; Farr *et al.*, 2004). Further supporting the hypothesis that

androstenedione might impair cognition via $GABA_AR$, androstanediol has been shown to be a positive allosteric modulator of $GABA_AR$ (Reddy & Jian, 2010). To assess androstenedione's impact on the GABAergic system, we measured levels of glutamic acid decarboxylase (GAD), the synthesizing enzyme and rate limiting step of GABA production, in multiple cognitive brain regions after maze testing concluded.

## Methods

### *Subjects*

Twenty-six fourteen month-old Fischer 344 female rats born and raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN) were used. Upon arrival, rats were pair housed, acclimated for two weeks before surgery, had access to food and water ad-lib and were maintained on a 12-hour light/dark cycle at the Arizona State University animal facility. All procedures were approved by the local IACUC committee and adhered to NIH standards.

### *Ovariectomy (OVX) and hormone treatment*

Approximately two weeks before behavioral testing, all rats received OVX under isofluorane inhalation. OVX consisted of bilateral dorsolateral incisions in the skin and peritoneum, the ovaries and tips of

the uterine horns were ligatured and removed, and muscle and skin were sutured. After surgery, rats received Rimadyl (5mg/mL/kg) for pain and saline (2mL) to prevent dehydration.

Rats were randomly assigned to one of three treatment groups: Vehicle (n=10), Androstenedione Low (n=8), and Androstenedione High (n=8) (Steraloids, Newport, RI, USA). Vehicle-treated animals received 0.5 mL of polyethylene glycol (PEG) (Sigma-Aldrich, St. Louis, MO, USA) only. The Androstenedione Low group was given 4mg/kg daily, and the Androstenedione High group was given 8mg/kg daily. Doses were based on previous literature (Lea & Flanagan, 1998; Sprando *et al.*, 2004), showing desired serum ranges of androstenedione comparable to the levels found in Acosta et. al (2009a) that were correlated with greater errors on a working memory task. Hormone or vehicle treatment began two days after surgery (12 days before behavioral testing ensued) and continued until sacrifice (See Figure 1 for timetable). The assigned substrate was administered daily between 0730 and 0830 via subcutaneous injection into the scruff of the neck. All hormone doses were dissolved in polyethylene glycol (PEG) so that each dose was given in 0.5mL PEG.

*Vaginal smears and uterine weights*

Vaginal smears were taken 8, 9, 10, 11, 28, and 44 days after OVX; hormone administration began 2 days after OVX, and thus hormone was being administered during the time when smears were done. Smears were classified as proestrus, estrous, metestrus, or diestrus, per prior protocols (Goldman *et al.*, 2007; Acosta *et al.*, 2009b). Prior studies have shown that androgens stimulate the uterus and lead to increased uterine weight (Ruizeveld de Winter *et al.*, 1991; Horie *et al.*, 1992). Thus, to further verify androstenedione's effects on uterine tissues, at sacrifice the uteri of all subjects were removed, trimmed of visible fat, and immediately weighed (wet weight).

*Water radial-arm maze (WRAM)*

Subjects were tested for 13 days on the eight-arm win-shift WRAM (Figure 2a) to evaluate spatial working and reference memory, including performance as working memory load increased, as described previously (Bimonte & Denenberg, 1999). The black plexiglass maze was filled with water made opaque with black non-toxic paint. The temperature of the water was 18°-19°C. The maze contained escape platforms hidden under the water surface in the ends of four of eight arms (each arm was 38.1 cm x 12.7 cm). Each subject was assigned different platform locations that remained constant throughout the 13 days of testing. The subjects were released from the start arm and had 3 minutes to locate a platform. Once

a platform was found, the subject remained on it for 15 seconds, and was then returned to its heated cage for a 30 second inter-trial interval (ITI) until its next trial. During the interval, the just located platform was removed from the maze and the water was stirred with a net to collect fecal boli and bedding. The animal was then placed again into the start alley, which remained constant throughout testing, and allowed to locate another platform. An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm and not visible from the inside of the maze (11cm into the arm). Orthogonal measures of working and reference memory errors were quantified as done previously in WRAM studies (Bimonte *et al.*, 2000). Working memory correct (WMC) errors were the number of first and repeat entries into an arm that previously contained a platform that session. Reference memory (RM) errors were the number of first entries into an arm that never contained a platform. Working memory incorrect (WMI) errors were repeat entries into reference memory arms. For each animal a daily session consisted of four trials, with the number of platformed arms reduced by one on each subsequent trial. Thus, the working memory system was increasingly taxed as trials progressed, allowing us to access performance as working memory load increased. Each subject was given one session a day for 12 consecutive days. After 12 days at a 30 second ITI, a 6 hour ITI was initiated between trials 2 and 3.

For WRAM analyses, for the three orthogonal measures of Working Memory Correct (WMC), Working Memory Incorrect (WMI), and Reference Memory (RM), learning across all testing days was evaluated in order to detect any Day x Treatment interactions. Learning during the acquisition phase (Days 2-6) was evaluated. Performance on the final two days of regular testing, Days 11-12, was evaluated at the highest working memory load (Trial 4), as this has revealed steroid hormone induced benefits, and age-related decrements, in our laboratory (Bimonte-Nelson *et al.*, 2003a; Braden *et al.*, 2010). For the 6 hour delay on Day 13, Treatment effects and Trial x Treatment interactions were analyzed for the post-delay trials (Trials 3 and 4) in order to detect potential delay-induced impairments across groups. Errors committed on the post-day trials were compared to errors on the last day of regular testing, Day 12 (baseline), in order to detect impairing effects of a 6 hour delay vs. the 30 second ITI within group.

*Delay-match-to-sample (DMS) three choice task*

Animals were tested for 7 days on the four arm win-stay DMS (Figure 2b) to evaluate spatial working memory and short-term memory retention. The black plexiglass maze (each arm was 38.1 cm x 12.7 cm) was filled with water made opaque with black non-toxic paint. The temperature of the water was 18°-19°C. Animals received 6 consecutive

trials a day for 6 days. The platform was located in a new arm each day, but remained in the same arm within a day for each subject. The drop off location varied for each trial, alternating between the three available arms two times each within a set of trials. Trial one was the information trial informing the animal where the platform was for that day's session, trial two was the working memory test trial and trials three through six were recent memory test trials (Frick *et al.*, 1995). Rats were given 90 seconds to find the platform. Once on the platform, the rat remained on it for 15 seconds, followed by placement into a heated cage for a 30 second ITI. An arm entry was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm and not visible from the inside of the maze (11cm into the arm). First errors were first entries into an arm without a platform, and Repeat errors were repeat entries into an arm without a platform within the same trial. Thus, the dependent variables were First errors, Repeat errors, and Total (First + Repeat errors) errors, for Days 1-6, Trials 2-6 (Trial 1 was the information trial). After 6 days of testing with a 30 second ITI, an increased time delay of 6 hours was given on day 7, between Trials 1 and 2, to assess delayed memory retention. Each error type was evaluated across all testing days in order to detect Day x Treatment interactions. Treatment effects were analyzed for the post-delay trial (Trial 2) in order to detect impairing effects of the delay within that day between treatment groups. Errors committed on the post-day trials were compared to errors on the last day of regular testing, Day 6

(baseline) in order to detect impairing effects of a 6 hour delay vs. the 30 second ITI within treatment groups.

*Morris water maze (MM)*

The MM maze (Figure 2c) was tested for six trials a day for 3 days using a tub (188 cm diameter) filled with black water made opaque using non-toxic paint. The temperature of the water was 18°-19°C. A hidden platform (10 cm wide) remained in a fixed location throughout testing, thereby testing spatial reference memory (Morris *et al.*, 1982; Bimonte-Nelson *et al.*, 2006). The subject was placed in the maze from the North, South, East, or West location (semi-randomly determined), and had 60 seconds to locate the hidden platform, located inside the Northeast quadrant throughout testing. Once the rat found the platform the trial was terminated. After 15 seconds of platform time, the subject was placed into its heated cage until its next trial. The approximate ITI was 15 minutes. To evaluate whether subjects localized the platform to the spatial location, after all test trials were completed on day 3, a 60 second probe trial was given whereby the platform was removed. For each trial, a camera suspended above the maze tracked each rat's path and a tracking system (Ethovision, Noldus Instruments, Leesburg, VA, USA) analyzed each rat's tracing.

MM performance was assessed by swim path distance (cm) to the platform. For probe trial data, total percent distance in the previously platformed (target) quadrant was compared to that in the quadrant diagonally opposite to the platform. Rats that learned the platform location were expected to swim the greatest distance in the target quadrant (Hyde *et al.*, 2002; Talboom *et al.*, 2008).

*Elevated plus maze (EPM)*

Animals were tested on the EPM (Figure 2d) for one day, with a 5 minute trial to evaluate anxiety, based on previously published protocols (Bellani *et al.*, 2006; Huynh *et al.*, 2011). The EPM consisted of four (50.8cm) contralateral arms (49.5 cm in length, 10.2cm in width) positioned in accordance to face North, South, East, and West. The closed arms (East and West arms) had walls surrounding the perimeters (50.8 cm in height), while the open arms had no walls surrounding the arm floors. Rats were placed into the center of the maze facing a randomly-assigned closed arm and given 5 minutes to explore the environment. For each trial, a camera suspended above the maze allowed for scoring on video. Time spent in the open arms, frequency of open arm entrances, frequency of closed arm entries, and total fecal boli were quantified. Time spent in the open arm, and open arm entrances, were defined as both front paws crossing into the open arm quadrant. Closed arm entrances

were defined as when both front paws crossing into the closed arm quadrant. At the end of the trial, the subject was placed back into its home cage, fecal boli were counted, and the maze was wiped clean with odor eliminator to clean any scents prior to the next trial. An added measure of anxiety was calculated for EPM using the following equation, which unified all EPM parameters into one unified ratio. Anxiety index values ranged from 0 to 1, with a higher value indicating increased anxiety. $$\text{Anxiety Index} = 1 - \left[\frac{(\text{open arm time}/5\text{ min}) + (\text{open arm entry}/\text{total entry})}{2}\right]$$

*Visible platform maze*

Since the MM, WRAM, and DMS rely on spatial navigation, it was necessary to confirm that all subjects had intact vision and could perform the procedural task components without difficulty. A visible platform water escape task was used. A rectangular tub (39 x 23 inches) was filled with clear water and a black platform (10 cm wide) was elevated above the water surface. Opaque curtains covered extramaze cues. The drop off location remained the same across trials, and the platform location for each trial varied in space semi-randomly. Animals had to locate the platform protruding from the water, and were given six trials for one day. Performance was assessed by latency (s) to the platform.

*Brain dissections*

Two days after the conclusion of behavior testing, animals were anesthetized with isofluorane, decapitated, brains rapidly dissected, and tissues frozen. Dissected tissues were stored in preweighed microcentrifuge tubes at -70°C until analysis. Dissections were performed according to plate designations in Paxinos and Watson (Paxinos & Watson, 1998) and were as follows: frontal cortex (plates 5-14), cingulate cortex (plates 5-14), basal forebrain (plates 14-16), perirhinal cortex (plates 39-42), entorhinal cortex (plates 39-42), and CA1/CA2 region of the dorsal hippocampus (plates 33-35). For each brain the frontal cortex was taken first from the dorsal aspect of the intact brain. Next, the cingulate cortex was taken with the longitudinal fissure as the medial border, and the medial border of the frontal cortex cut as the lateral border. The brain was then cut in the coronal plane to obtain access to the basal forebrain. For the basal forebrain, both medial septum and ventral diagonal band were included with the posterior landmark being the crossing point of the anterior commissure. The brains were then cut in the coronal plane to obtain access to the last three brain regions. For the CA1/CA2 region of the hippocampus, dentate gyrus and the alveus were excluded. For the entorhinal cortex, the tissue was dissected from the same slice as the hippocampal sample, taking a 2- to 3-mm sample ventral to the hippocampus. The perirhinal cortex was also collected from this same slice, taking a 2- to 3-mm sample around the perirhinal fissure.

*Western blot analysis of GAD 65 + 67*

GAD 65 + 67 protein expression levels were analyzed in frontal, cingulate, perirhinal and entorhinal cortices, and hippocampus, from the right hemisphere, as well as the basal forebrain. Samples were sonicated in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na Deoxycholate, 50 mM Tris) equivalent to 10 times the weight of the sample and centrifuged at 10,000 RPM for 10 m at 4°C. Protein concentrations were determined using BCA protein assays (ThermoFischer Scientific, Pittsburgh, PA, USA) and 10 µg of protein from each sample was run on a Run Blue 4-12% SDS gel, using the SureLock mini-cell (Invitrogen, Carlsbad, CA, USA). Gels were counterbalanced by group and each region was run in two gels. Half of each group was loaded onto a single gel, with two gels total for each brain region. Proteins were transferred onto a PVDF membrane (Millipore, Bedford, MA, USA) and immunoblotting was performed with working dilutions of rabbit anti-GAD 65 + 67 (ab11070) and rabbit anti-beta Actin (ab25894) primary antibodies (Abcam Inc., Cambridge, MA, USA). Antibody dilutions were 1:10,000 for GAD 65 + 67 and 1:1,000 for beta Actin primary antibodies. Membranes were then exposed to a peroxidase-conjugated goat anti-rabbit secondary antibody, 1:20,000 dilution (111-035-003; Jackson Immuno Research, West Grove, PA, USA) and visualized using Pierce ECL (ThermoScientific, Rockford, IL, USA) on a Biospectrum Biochemi 500 Imaging System (UVP, Upland, CA, USA). Bands were identified as

the protein of interest, based on molecular weight, using Precision Plus Protein WesternC Standards (Bio Rad, Hercules, CA, USA). The density of GAD 65 + 67 and beta Actin (control protein) was then quantified using ImageJ software (Rasband, 1997-2004). The dependent measure was a proportion of each subject's GAD 65 + 67 density to their beta Actin density, brought to percent control of Vehicle subjects run on the same gel, per prior protocols (Pandey *et al.*, 1999; Braden *et al.*, 2010; Braden *et al.*, 2011)

*Blood serum analysis*

At the time of brain dissection, blood was collected via cardiac puncture (Vacutainer 367986, Becton Dickinson and Company, Franklin Lakes, NJ). Blood was allowed to clot at 4°C and serum was collected after centrifugation (3220, 20min). Serum was stored at -20°C until androstenedione assays were performed by the Core Endocrinology Laboratory at Pennsylvania State University College of Medicine. Serum levels of androstenedione were determined by iondinated RIA using reagents obtained from Siemens (Los Angeles, CA, USA) as done previously (Acosta *et al.*, 2010). The low-end sensitivity for this androstenedione method is 0.1 ng/ml.

*Statistical analyses*

Data were analyzed separately for each maze. WRAM, DMS, MM, and Visible Platform were first analyzed with a repeated measures ANOVA with Treatment as the between variable and Days and/or Trials as the within variable, as appropriate for the specific maze test. EPM, GAD western blots, uterine weights, and serum analyses were analyzed using t-tests with Treatment as the between variable. Since our interest was to determine whether each dose of androstenedione enhanced or impaired performance relative to the Vehicle group, all of our comparisons were planned. Any analysis comparing the Androstenedione Low to Androstenedione High group was done post hoc.

Correlations were used to examine relationships between androstenedione serum levels and behavioral results found to have significance using the ANOVA tests described above. Circulating androstenedione levels were correlated with the following outcome variables: MM distance collapsed across all trials/days; DMS Total Errors postdelay; WRAM RM errors Days 2-6 Trial 2; and WRAM WMI errors Days 11-12 Trial 4. Correlations were also used to examine relationships between GAD levels in multiple brain regions with the same behavioral results assessed in the androstenedione serum correlation analysis stated above.

## Results

### *Vaginal smears and uterine weights*

During hormone treatment, all Vehicle and Androstenedione Low animals showed continuous diestrus smears, indicating lack of uterine stimulation, while Androstenedione High animals showed vaginal smears with an increased amount of cornified cells, indicating uterine stimulation.

Uteri of Androstenedione High animals weighed more than those of all other treatment groups (Androstenedione High vs. Vehicle: $t_{16} = 2.47$; $P = 0.025$; Androstenedione High vs. Androstenedione Low: $t_{14} = 2.526$; $P = 0.024$), as shown in Figure 3.

### *Water radial-arm maze*

There were no treatment main effects or interactions for WMC, WMI, RM during the acquisition phase (Days 2-6). However, upon inspection of the acquisition curves, we noted group differences for RM errors specific to Trial 2. RM errors on the WRAM maze are capped at a maximum of 4 errors each, with one entry per unplatformed arm counted as a reference memory error. Thus, committing errors on earlier trials limits the number of errors that can be made on later trials, and errors on earlier trials can be interpreted as earlier reference memory failure. In this regard, we analyzed Trial 2 alone post-hoc. The Androstenedione High

group committed more RM errors compared to both Vehicle ($F_{1,16}$ = 6.974; $P$ = 0.018) and Androstenedione Low ($F_{1,14}$ = 10.654; $P$ = 0.006) groups (Figure 4a). There were no treatment main effects or interactions for WMC or RM errors on Trial 4 during the final two days of testing (Days 11-12). There was a main effect for WMI errors on these final two days of testing, where the Androstenedione High group committed more WMI errors compared to both the Vehicle group ($F_{1,16}$ = 6.673; $P$ = 0.018) and Androstenedione Low group ($F_{1,14}$ = 7.862; $P$ = 0.014) (Figure 4b). There were no treatment main effects or interactions for WMC, WMI, or RM for the 6-hour delay given on Day 13.

*Delay-match-to-sample three choice task*

There were no treatment effects for Total Errors, First Errors, or Repeat Errors for Days 1-6. The high dose androstenedione treatment impaired retention on the 6-hour delay manipulation given between trials 1 and 2. For the 6-hour delay given on day 7, Androstenedione High was the only group to commit significantly more Total Errors (Figure 5) ($t_7$ = 3.862; $P$ = 0.006) and First Errors ($t_7$ = 4.245; $P$ = 0.004) compared to baseline (trial 2 on day 6). Androstenedione Low and Vehicle groups were not impaired by the 6-hour delay, as they did not differ from their baseline scores to their delay scores.

*Morris water maze*

Figure 6 shows the mean distance to platform scores ± SE for each treatment group across the three days of MM testing, collapsed across trials. Androstenedione treatment at the high dose impaired spatial reference memory; Androstenedione High rats swam a greater distance to the platform compared to Vehicle rats ($F_{1,16}$ = 4.871;P = 0.042). There was also a significant linear trend for dose of androstenedione ($F_{1,2}$ = 182.905; $P$ = 0.047), with swim distance increasing as dose of androstenedione increased. For the probe trial, each treatment group swam a higher percent swim distance in the target quadrant versus the opposite quadrant [Androstenedione High ($t_7$ = 5.693; $P$ = .001), Androstenedione Low ($t_7$ = 5.565; $P$ = .001), Vehicle ($t_9$ = 10.336; $P$ < .0001)], indicating that all groups, regardless of hormone status, localized the platform quadrant by the end of testing. There were no group differences for platform crossings.

*Elevated plus maze*

There were no treatment main effects for number of open arm entries (Figure 7a), number of closed arm entries (data not shown), amount of time spent in open arms (Figure 7b), and number of fecal boli (data not shown). As well, there were no treatment main effects for the Anxiety Index (date not shown).

*Visible platform*

There were no Treatment main effects or interactions for latency on the visible platform task (Figure 8). There was a Trial main effect ($F_{2,5}$ = 11.172; $P < .0001$), with Time decreasing across all trials of testing. By the last trial of testing, all subjects found the visible platform within 10 seconds. These data confirm visual and motor competence to perform swim maze tasks.

*Serum androstenedione levels*

As expected, each Vehicle rat had undetectable levels of serum androstenedione. The Androstenedione High group had higher serum androstenedione levels than the Vehicle group ($t_{16}$ = 4.282; $P$ = .0006), and the Androstenedione Low group had higher serum levels than the Vehicle group ($t_{16}$ = 3.846; $P$ = .0014) (Figure 9a).

As shown in Figure 9b, replicating our prior correlations (Acosta *et al.*, 2009a; Acosta *et al.*, 2010), there was a significant correlation between androstenedione serum levels and WRAM WMI errors Days 11-12 Trial 4 ($r_{25}$ = .609; $P$ = .0007), with higher androstenedione levels correlating with worse performance. There were no significant correlations for androstenedione serum levels and MM distance collapsed across all trials/days; DMS Total errors post delay; and WRAM RM errors Days 2-6 Trial 2.

*Western Blots*

There were no Treatment effects for GAD levels for the frontal cortex, cingulate cortex, basal forebrain, hippocampus, entorhinal cortex, or perirhinal cortex (Table 1). There were also no Treatment effects for the loading control, beta actin. For correlation analyses, all data points were centered to control for group differences, as done previously (Talboom *et al.*, 2010); for more detailed methods and rationale of this procedure see (Hallahan & Rosenthal, 2000; Enders & Tofighi, 2007). There was a significant correlation between GAD levels in the entorhinal cortex and MM distance collapsed across all days and trials. Animals that had higher GAD levels in the entorhinal cortex tended to swim a greater distance on the MM ($r_{23}$ = .431; *P* = .036), as seen in Figure 10, suggesting that higher GAD levels in the entorhinal cortex are associated with poorer spatial reference memory performance.

## Discussion

The current study is the first to methodically evaluate androstenedione's effects on learning and memory in the middle-aged female rodent. Here, we demonstrate that androstenedione administration impairs performance within multiple domains of cognitive function. Specifically, in middle-aged OVX rats, androstenedione impaired spatial reference memory as well as the ability to maintain performance with

increasing memory demand. This was true for both high demand memory retention of one item of spatial information (as seen on the DMS task) as well as the ability to handle multiple items of spatial working memory information (as seen on the WRAM task). The lack of group differences on the visible platform task and elevated plus maze suggest that the cognitive deficits observed on spatial reference and working memory were not attributed to visual impairment or anxiety. We also confirmed androgen-induced uterine weight increases, as well as circulating androstenedione serum blood levels. Indeed, consistent with previous findings showing uterine weight increases after treatment with other androgens (Nantermet et al., 2005), androstenedione treatment elevated uterine weights at the highest dose, relative to vehicle treatment, in the current study. Androgens appear to elevate uterine weight via direct action of androgen receptors located in the uterus (Ruizeveld de Winter *et al.*, 1991; Horie *et al.*, 1992), an effect not reversed by estrogen receptor antagonists (Schmidt & Katzenellenbogen, 1979), thereby suggesting that androstenedione effects on the uterus may be due to its androgenic actions rather than from aromatized estrogens. Serum evaluation showed that androstenedione levels were higher in both the low and high dose groups relative to vehicle, and every rat in the vehicle group produced serum androstenedione levels that were undetectable; collectively, these results confirm that only the rats in the androstenedione low and high groups had the hormone in circulation at levels detectable by the assay used herein.

Importantly, both administered doses of androstenedione produced blood serum levels comparable to the range of levels achieved in the Acosta et al. (2009a) study. This allows for a controlled cognitive analysis of androstenedione at the levels produced in the transitional model, while also controlling for any interaction that may occur with presence of the residual ovary and any other hormones secreted.

Androstenedione dose-dependently negatively impacted spatial reference memory on the Morris maze, with a greater swim distance to the platform as the dose increased. It is noteworthy that this impairment pervades across all trials and all days of traditional testing; yet, there were no group differences in the probe trial. Thus, despite androstenedione-induced impairments at the high dose on the MM across the three test days, rats caught up by the end of testing and eventually learned the task. This impairment in learning spatial reference memory was also observed in the WRAM. Rats receiving the high dose of androstenedione committed more RM errors compared to those receiving the low dose of androstenedione or the vehicle on Trial 2 during the acquisition phase of testing (Days 2-6). This difference was not shown in the second block of testing (Days 7-12), indicating that rats receiving the high androstenedione dose eventually learned the task similar to rats receiving the low dose or vehicle.

The current study also found that androstenedione impaired the ability to maintain performance as memory demand was increased. This

was true for both high demand memory retention of one item of spatial information, as well as the ability to handle multiple items of spatial working memory information. On the final two days of testing for WRAM, the rats receiving the high dose of androstenedione committed more WMI errors on Trial 4, when the working memory load was at its highest, compared to those receiving the low dose of androstenedione or vehicle. This androstenedione-induced impairment was also shown in the significant correlation between WRAM WMI errors on Trial 4 on the last two days of testing and androstenedione serum levels. This is consistent with the results from Acosta et al. 2009a, where there was a significant correlation between WMI errors and androstenedione serum level with more errors correlating with higher circulating androstenedione levels. On the DMS 6 hour delay, there was a high demand for memory retention for one item of spatial information. We found that the rats receiving the androstenedione high dose were the only group to commit significantly greater errors after the delay compared to baseline. Taken together, these findings suggest that androstenedione at a high physiological level impairs the ability to handle learning and memory tasks that require greater memory demand.

The herein study suggests that $GABA_AR$ are not involved in androstenedione's detrimental impact on cognition, as we failed to detect hormone treatment-related group differences in GAD levels in multiple brain regions. We did, however, find a correlation between GAD levels in

the entorhinal cortex and swim distance to the platform in the Morris water maze when collapsed across all treatment groups. Specifically, animals that had higher GAD levels in the entorhinal cortex tended to swim a greater distance on the MM, thereby suggesting that higher GAD levels in the entorhinal cortex are associated with poorer spatial reference memory performance. This is a replication of our previous work showing an increase in GAD levels in the entorhinal cortex and impairment in spatial reference memory (Braden et al., 2010). The entorhinal cortex and the hippocampus are associated with, and play crucial roles in, memory processing (Knowles, 1992), which increasing evidence suggests is facilitated in part through the GABAergic system (Izquierdo *et al.*, 1993). However, the direct mechanism via which androstenedione exerts its effects remain to be elucidated.

Administration of androstenedione may set forth a cascade of events leading to a disruption of neuroprotective events through either direct action on the androgen receptor, or through its conversion to estrone. Indeed, while the estrogens 17β-estradiol and equilin have been found to be neuroprotective (Vegeto *et al.*, 2002; Zhao & Brinton, 2006), estrone was found to not have the same neuroprotective effects (Zhao & Brinton, 2006). Thus, estrone's lack of neuroprotective abilities may be playing a role the impairments of memory found in this study. Androstenedione may also be impacting cognition through its actions on the androgen receptor via its conversion to testosterone. Indeed,

androgen receptors have been located in brain regions important for cognition, such as the hippocampus, amygdala, and cortex (Barley *et al.*, 1975; Sheridan, 1983; Handa *et al.*, 1986; Hajszan *et al.*, 2008). Future research aimed at finding the mechanism(s) responsible for androstenedione-induced cognitive impairment could yield valuable insight into the hormonal mechanisms relating to the negative cognitive consequences of menopause, whereby follicular depletion occurs along with retention of the residual ovary, shown in the VCD animal model. Future studies to gain insight might include methodically evaluating the specific contributions of androstenedione to cognition in female rodent models via pharmaceutical manipulations of androgenic stimulation by receptor or enzymatic blockade.

Although androstenedione has not been previously evaluated for spatial cognition in older rats, other androgens have been assessed. The most commonly tested androgens have been dihydrotestosterone, testosterone, and dehydroepiandrosterone. Research suggests that dihydrotestosterone has no impact on spatial reference memory or working memory (Raber *et al.*, 2002; Bimonte-Nelson *et al.*, 2003b; Benice & Raber, 2009). On the contrary, we and other have shown that testosterone treatment has been shown to improve working memory (Bimonte-Nelson *et al.*, 2003b), spatial reference memory (Benice & Raber, 2009), and avoidance learning and memory (Flood *et al.*, 1995; Edinger *et al.*, 2004). The difference between androstenedione's negative

cognitive impact, and testosterone's positive cognitive impact, may be related to the type of estrogen to which these two androgens directly synthesize. Testosterone is directly aromatized to estradiol, while androstenedione is directly aromatized to estrone. It may be possible that the androstenedione-induced cognitive impairment is in part mediated through its conversion to estrone; indeed, androstenedione administration significantly increases estrone serum levels (Jasuja *et al.*, 2005). The administration of estrone exogenously appears to negatively affect cognition; however, this has only been examined in two studies thus far. Work done thus far has found that administration of estrone impaired contextual fear conditioning in young adult female rats (Barha *et al.*, 2010) as well as working memory on a delay-match-to-sample win-stay task in middle-aged female rats (Engler-Chiurazzi, in review). Research looking at the effects of estrone administration on middle-aged and aged rats, along with assessing a larger battery of cognitive tasks is needed to gain a better understanding of estrone's impact on cognition.

In conclusion, the current study is the first to methodically evaluate androstenedione's effects on learning and memory in the middle-aged female rodent model. Here, we demonstrate that androstenedione treatment impaired spatial reference memory, as well as the ability to maintain performance as memory demand was elevated. These results, taken with prior findings that higher androstenedione levels correlate with poorer working memory in the follicle deplete reproductively senescent

ovary intact rat (Acosta *et al.*, 2009a), indicate that androstenedione is a key variable impacting memory abilities as menopause ensues. Understanding the mechanisms through which androstenedione is impairing memory may reveal valuable information toward optimization of factors during menopause that could influence cognition.

# REFERENCES


**Acosta, J.I., Mayer, L., Talboom, J.S., Tsang, C.W., Smith, C.J., Enders, C.K. & Bimonte-Nelson, H.A. (2009a) Transitional versus surgical menopause in a rodent model: etiology of ovarian hormone loss impacts memory and the acetylcholine system. *Endocrinology*.**

**Acosta, J.I., Mayer, L., Talboom, J.S., Zay, C., Scheldrup, M., Castillo, J., Demers, L.M., Enders, C.K. & Bimonte-Nelson, H.A. (2009b) Premarin improves memory, prevents scopolamine-induced amnesia and increases number of basal forebrain choline acetyltransferase positive cells in middle-aged surgically menopausal rats. *Horm Behav*, 55, 454-464.**

**Acosta, J.I., Mayer, L.P., Braden, B.B., Nonnenmacher, S., Mennenga, S.E. & Bimonte-Nelson, H.A. (2010) The cognitive effects of conjugated equine estrogens depend on whether menopause etiology is transitional or surgical. *Endocrinology*, 151, 3795-3804.**

**Barha, C.K., Dalton, G.L. & Galea, L.A. (2010) Low doses of 17alpha-estradiol and 17beta-estradiol facilitate, whereas higher doses of estrone and 17alpha- and 17beta-estradiol impair, contextual fear conditioning in adult female rats. *Neuropsychopharmacology*, 35, 547-559.**

**Barley, J., Ginsburg, M., Greenstein, B.D., MacLusky, N.J. & Thomas, P.J. (1975) An androgen receptor in rat brain and pituitary. *Brain Res*, 100, 383-393.**

**Bellani, R., Luecken, L.J. & Conrad, C.D. (2006) Peripubertal anxiety profile can predict predisposition to spatial memory impairments following chronic stress. *Behav Brain Res*, 166, 263-270.**

**Benice, T.S. & Raber, J. (2009) Testosterone and dihydrotestosterone differentially improve cognition in aged female mice. *Learn Mem*, 16, 479-485.**

**Bimonte-Nelson, H.A., Francis, K.R., Umphlet, C.D. & Granholm, A.C. (2006) Progesterone reverses the spatial memory enhancements initiated by tonic and cyclic oestrogen therapy in middle-aged ovariectomized female rats. *Eur J Neurosci*, 24, 229-242.**

**Bimonte-Nelson, H.A., Singleton, R.S., Hunter, C.L., Price, K.L., Moore, A.B. & Granholm, A.C. (2003a) Ovarian hormones and cognition in the aged female rat: I. Long-term, but not short-term, ovariectomy enhances spatial performance. *Behav Neurosci*, 117, 1395-1406.**

**Bimonte-Nelson, H.A., Singleton, R.S., Nelson, M.E., Eckman, C.B., Barber, J., Scott, T.Y. & Granholm, A.C. (2003b) Testosterone, but not**

**nonaromatizable dihydrotestosterone, improves working memory and alters nerve growth factor levels in aged male rats. *Exp Neurol*, 181, 301-312.**

**Bimonte, H.A. & Denenberg, V.H. (1999) Estradiol facilitates performance as working memory load increases. *Psychoneuroendocrinology*, 24, 161-173.**

**Bimonte, H.A., Hyde, L.A., Hoplight, B.J. & Denenberg, V.H. (2000) In two species, females exhibit superior working memory and inferior reference memory on the water radial-arm maze. *Physiol Behav*, 70, 311-317.**

**Birzniece, V., Turkmen, S., Lindblad, C., Zhu, D., Johansson, I.M., Backstrom, T. & Wahlstrom, G. (2006) GABA(A) receptor changes in acute allopregnanolone tolerance. *Eur J Pharmacol*, 535, 125-134.**

**Braden, B.B., Garcia, A.N., Mennenga, S.E., Prokai, L., Villa, S.R., Acosta, J.I., Lefort, N., Simard, A.R. & Bimonte-Nelson, H.A. (2011) Cognitive-impairing effects of medroxyprogesterone acetate in the rat: independent and interactive effects across time. *Psychopharmacology (Berl)*.**

**Braden, B.B., Talboom, J.S., Crain, I.D., Simard, A.R., Lukas, R.J., Prokai, L., Scheldrup, M.R., Bowman, B.L. & Bimonte-Nelson, H.A. (2010) Medroxyprogesterone acetate impairs memory and alters the GABAergic system in aged surgically menopausal rats. *Neurobiol Learn Mem*, 93, 444-453.**

**Brioni, J.D., Nagahara, A.H. & McGaugh, J.L. (1989) Involvement of the amygdala GABAergic system in the modulation of memory storage. *Brain Res*, 487, 105-112.**

**Castellano, C., Introini-Collison, I.B. & McGaugh, J.L. (1993) Interaction of beta-endorphin and GABAergic drugs in the regulation of memory storage. *Behav Neural Biol*, 60, 123-128.**

**Castellano, C. & McGaugh, J.L. (1990) Effects of post-training bicuculline and muscimol on retention: lack of state dependency. *Behav Neural Biol*, 54, 156-164.**

**Edinger, K.L., Lee, B. & Frye, C.A. (2004) Mnemonic effects of testosterone and its 5alpha-reduced metabolites in the conditioned fear and inhibitory avoidance tasks. *Pharmacol Biochem Behav*, 78, 559-568.**

**Enders, C.K. & Tofighi, D. (2007) Centering predictor variables in cross-sectional multilevel models: a new look at an old issue. *Psychol Methods*, 12, 121-138.**

**Engler-Chiurazzi, E., Talboom, J. S., Braden, B.B., Tsang, C.W.S., Mennenga, S., Andrews, M., Demers, L.M., Bimonte-Nelson, H. A. (in**

**review) Tonic estrone treatment impairs spatial memory and does not impact number of**
**basal forebrain cholinergic neurons in the surgically menopausal middle-aged rat. *Horm Behav*, [Research Article].**

**Farr, S.A., Banks, W.A., Uezu, K., Gaskin, F.S. & Morley, J.E. (2004) DHEAS improves learning and memory in aged SAMP8 mice but not in diabetic mice. *Life Sci*, 75, 2775-2785.**

**Farrag, A.K., Khedr, E.M., Abdel-Aleem, H. & Rageh, T.A. (2002) Effect of surgical menopause on cognitive functions. *Dement Geriatr Cogn Disord*, 13, 193-198.**

**Flood, J.F., Farr, S.A., Kaiser, F.E., La Regina, M. & Morley, J.E. (1995) Age-related decrease of plasma testosterone in SAMP8 mice: replacement improves age-related impairment of learning and memory. *Physiol Behav*, 57, 669-673.**

**Flood, J.F. & Roberts, E. (1988) Dehydroepiandrosterone sulfate improves memory in aging mice. *Brain Res*, 448, 178-181.**

**Frick, K.M., Baxter, M.G., Markowska, A.L., Olton, D.S. & Price, D.L. (1995) Age-related spatial reference and working memory deficits assessed in the water maze. *Neurobiol Aging*, 16, 149-160.**

**Goldman, J.M., Murr, A.S. & Cooper, R.L. (2007) The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Res B Dev Reprod Toxicol*, 80, 84-97.**

**Grodin, J.M., Siiteri, P.K. & MacDonald, P.C. (1973) Source of estrogen production in postmenopausal women. *J Clin Endocrinol Metab*, 36, 207-214.**

**Hajszan, T., MacLusky, N.J. & Leranth, C. (2008) Role of androgens and the androgen receptor in remodeling of spine synapses in limbic brain areas. *Horm Behav*, 53, 638-646.**

**Hallahan, M. & Rosenthal, R. (2000) Interpreting and reporting results. In Tinsley, H.E.A., Brown, S.D. (eds) *Handbook of multivariate statistics and mathematical modeling*. Academic Press, San Diego, CA, pp. 183-208.**

**Handa, R.J., Reid, D.L. & Resko, J.A. (1986) Androgen receptors in brain and pituitary of female rats: cyclic changes and comparisons with the male. *Biol Reprod*, 34, 293-303.**

**Horie, K., Takakura, K., Imai, K., Liao, S. & Mori, T. (1992) Immunohistochemical localization of androgen receptor in the human endometrium, decidua, placenta and pathological conditions of the endometrium. *Hum Reprod*, 7, 1461-1466.**

**Huynh, T.N., Krigbaum, A.M., Hanna, J.J. & Conrad, C.D. (2011) Sex differences and phase of light cycle modify chronic stress effects on anxiety and depressive-like behavior. *Behav Brain Res*, 222, 212-222.**

**Hyde, L.A., Stavnezer, A.J., Bimonte, H.A., Sherman, G.F. & Denenberg, V.H. (2002) Spatial and nonspatial Morris maze learning: impaired behavioral flexibility in mice with ectopias located in the prefrontal cortex. *Behav Brain Res*, 133, 247-259.**

**Izquierdo, I., Medina, J.H., Bianchin, M., Walz, R., Zanatta, M.S., Da Silva, R.C., Bueno e Silva, M., Ruschel, A.C. & Paczko, N. (1993) Memory processing by the limbic system: role of specific neurotransmitter systems. *Behav Brain Res*, 58, 91-98.**

**Jasuja, R., Ramaraj, P., Mac, R.P., Singh, A.B., Storer, T.W., Artaza, J., Miller, A., Singh, R., Taylor, W.E., Lee, M.L., Davidson, T., Sinha-Hikim, I., Gonzalez-Cadavid, N. & Bhasin, S. (2005) Delta-4-androstene-3,17-dione binds androgen receptor, promotes myogenesis in vitro, and increases serum testosterone levels, fat-free mass, and muscle strength in hypogonadal men. *J Clin Endocrinol Metab*, 90, 855-863.**

**Knowles, W.D. (1992) Normal anatomy and neurophysiology of the hippocampal formation. *J Clin Neurophysiol*, 9, 252-263.**

**Lea, C.K. & Flanagan, A.M. (1998) Physiological plasma levels of androgens reduce bone loss in the ovariectomized rat. *Am J Physiol*, 274, E328-335.**

**Mayer, L.P., Devine, P.J., Dyer, C.A. & Hoyer, P.B. (2004) The follicle-deplete mouse ovary produces androgen. *Biol Reprod*, 71, 130-138.**

**Mayer, L.P., Pearsall, N.A., Christian, P.J., Devine, P.J., Payne, C.M., McCuskey, M.K., Marion, S.L., Sipes, I.G. & Hoyer, P.B. (2002) Long-term effects of ovarian follicular depletion in rats by 4-vinylcyclohexene diepoxide. *Reprod Toxicol*, 16, 775-781.**

**Meites, J. & Lu, J.K.H. (1994) Reproductive aging and neuroendocrine function. In Charlton, H.M. (ed) *Oxford review of reproductive biology* Oxford Press, New York.**

**Morris, R.G., Garrud, P., Rawlins, J.N. & O'Keefe, J. (1982) Place navigation impaired in rats with hippocampal lesions. *Nature*, 297, 681-683.**

**Nantermet, P.V., Masarachia, P., Gentile, M.A., Pennypacker, B., Xu, J., Holder, D., Gerhold, D., Towler, D., Schmidt, A., Kimmel, D.B., Freedman, L.P., Harada, S. & Ray, W.J. (2005) Androgenic induction of growth and differentiation in the rodent uterus involves the modulation of estrogen-regulated genetic pathways. *Endocrinology*, 146, 564-578.**

**Nappi, R.E., Sinforiani, E., Mauri, M., Bono, G., Polatti, F. & Nappi, G. (1999) Memory functioning at menopause: impact of age in ovariectomized women. *Gynecol Obstet Invest*, 47, 29-36.**

**Pandey, S.C., Zhang, D., Mittal, N. & Nayyar, D. (1999) Potential role of the gene transcription factor cyclic AMP-responsive element binding protein in ethanol withdrawal-related anxiety. *J Pharmacol Exp Ther*, 288, 866-878.**

**Paxinos, G. & Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates, 4 ed.* Academic Press, New York.**

**Raber, J., Bongers, G., LeFevour, A., Buttini, M. & Mucke, L. (2002) Androgens protect against apolipoprotein E4-induced cognitive deficits. *J Neurosci*, 22, 5204-5209.**

**Reddy, D.S. & Jian, K. (2010) The testosterone-derived neurosteroid androstanediol is a positive allosteric modulator of GABAA receptors. *J Pharmacol Exp Ther*, 334, 1031-1041.**

**Ruizeveld de Winter, J.A., Trapman, J., Vermey, M., Mulder, E., Zegers, N.D. & van der Kwast, T.H. (1991) Androgen receptor expression in human tissues: an immunohistochemical study. *J Histochem Cytochem*, 39, 927-936.**

**Schmidt, W.N. & Katzenellenbogen, B.S. (1979) Androgen-uterine interactions: an assessment of androgen interaction with the testosterone- and estrogen-receptor systems and stimulation of uterine growth and progesterone-receptor synthesis. *Mol Cell Endocrinol*, 15, 91-108.**

**Sheridan, P.J. (1983) Androgen receptors in the brain: what are we measuring? *Endocr Rev*, 4, 171-178.**

**Sprando, R.L., Collins, T.F., Black, T.N., Olejnik, N., Grundel, E. & Ruggles, D.I. (2004) Effects of androstenedione on in utero development in rats. *Food Chem Toxicol*, 42, 917-924.**

**Talboom, J.S., Engler-Chiurazzi, E.B., Whiteaker, P., Simard, A.R., Lukas, R., Acosta, J.I., Prokai, L. & Bimonte-Nelson, H.A. (2010) A component of Premarin((R)) enhances multiple cognitive functions and influences nicotinic receptor expression. *Horm Behav*, 58, 917-928.**

**Talboom, J.S., Williams, B.J., Baxley, E.R., West, S.G. & Bimonte-Nelson, H.A. (2008) Higher levels of estradiol replacement correlate with better spatial memory in surgically menopausal young and middle-aged rats. *Neurobiol Learn Mem*.**

**Timaras, P., Quay, W. & vernadakis, A. (eds) (1995) *Hormones and aging*. CRC Press, Boca Raton, NY, London, Tokyo.**

**Vegeto, E., Ciana, P. & Maggi, A. (2002) Estrogen and inflammation: hormone generous action spreads to the brain. *Mol Psychiatry*, 7, 236-238.**

**Yonkov, D., Georgiev, V., Kambourova, T. & Opitz, M. (1987) Participation of angiotensin II in learning and memory. III. Interactions of angiotensin II with GABAergic drugs. *Methods Find Exp Clin Pharmacol*, 9, 205-208.**

**Yonkov, D.I. & Georgiev, V.P. (1985) Memory effects of GABA-ergic antagonists in rats trained with two-way active avoidance tasks. *Acta Physiol Pharmacol Bulg*, 11, 44-49.**

**Zarrindast, M.R., Shamsi, T., Azarmina, P., Rostami, P. & Shafaghi, B. (2004) GABAergic system and imipramine-induced impairment of memory retention in rats. *Eur Neuropsychopharmacol*, 14, 59-64.**

**Zhao, L. & Brinton, R.D. (2006) Select estrogens within the complex formulation of conjugated equine estrogens (Premarin) are protective against neurodegenerative insults: implications for a composition of estrogen therapy to promote neuronal function and prevent Alzheimer's disease. *BMC Neurosci*, 7, 24.**

# APPENDIX A

## IACUC APPROVAL

**INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE AT ARIZONA STATE UNIVERSITY**

Certifies that

# Bryan Camp

as of this date

6/27/2012

Has completed animal care training for

IACUC Basics & Rat

Certificate Number

5626

APPENDIX B

# TABLES AND FIGURES

*Figure 1*. Schematic representation of the experiment.

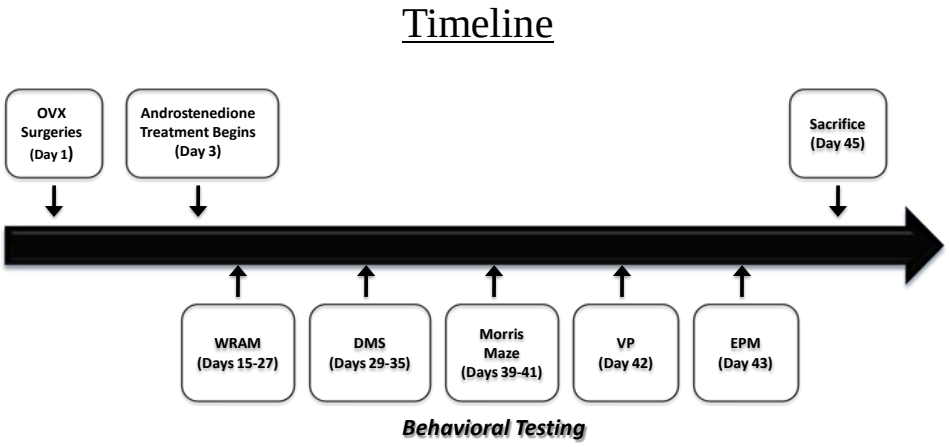

*Figure 2*. Diagrams of the behavioral mazes used in the experiment. **a.** Water Radial Arm Maze. **b.** Delay Match to Sample Three Choice Task. **c.** Morris Water Maze. **d.** Elevated Plus Maze.

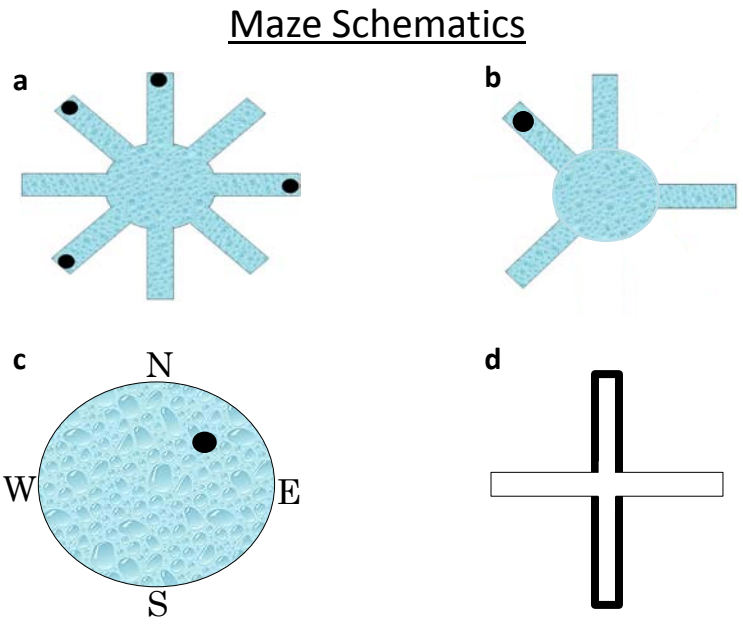

*Figure 3*. Mean (±SE) uterine weights for each group. Uterine weights of the Androstenedione High group were greater than those in both the Androstenedione Low group [$t(14) = 2.47$; $P = 0.025$] and the Vehicle group [$t(16) = 2.526$; $P = 0.024$].

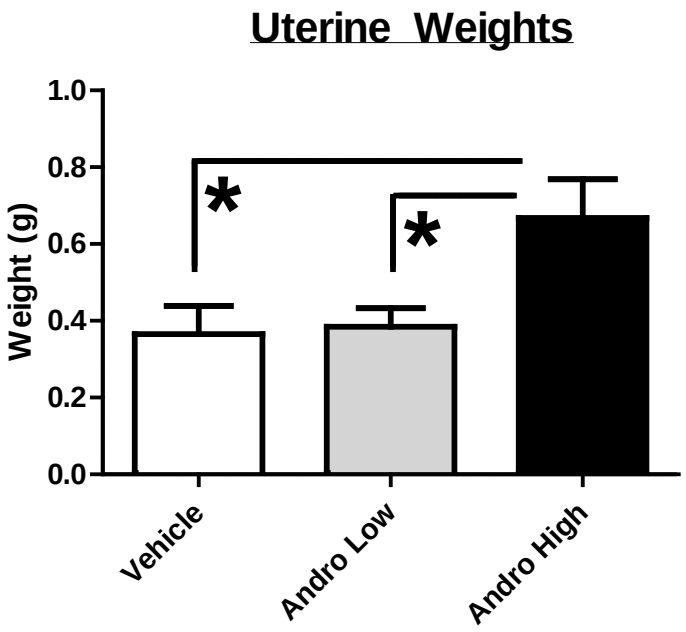

*Figure 4*. **a.** Mean (±SE) Reference Memory Errors on the WRAM during the acquisition phase (Days 2-6) on Trial 2. Androstenedione High group committed significantly more errors compared to both the Androstenedione Low group [$F(1,14) = 10.654$; $P = 0.006$] and the Vehicle group [$F(1,16) = 6.974$; $P = 0.018$]. **b.** Mean (±SE) Working Memory Incorrect Errors on the WRAM during the Trial 4 on the last two days of testing (Days 11-12). The Androstenedione High group committed significantly more errors compared to both the Androstenedione Low group [$F(1,14) = 7.862$; $P = 0.014$] and the Vehicle group [$F(1,16) = 6.673$; $P = 0.02$].

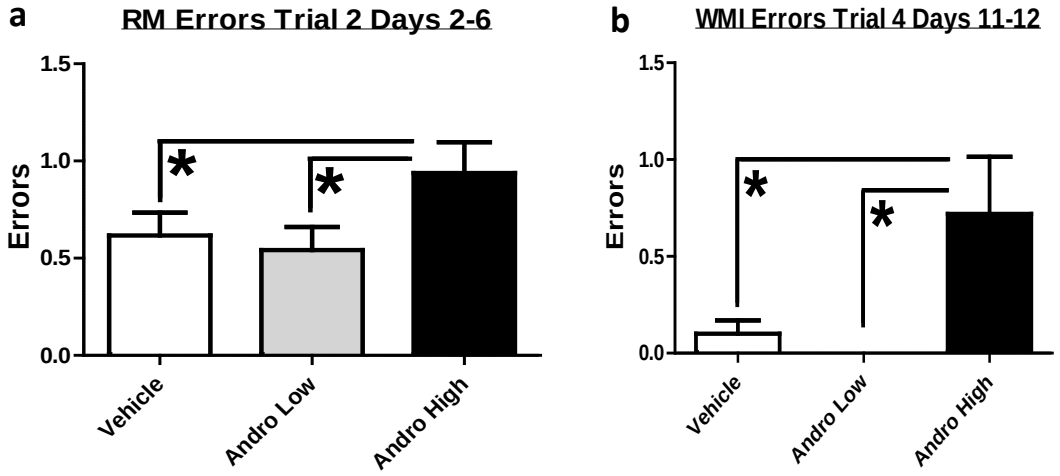

*Figure 5.* Mean (±SE) Total Errors for each group for post-delay trials on the DMS. The Androstenedione High group was the only group to make significantly more post-delay errors following the 6 hour delay between trials 1 and 2 [$t(7) = 3.862$; $P = 0.006$].

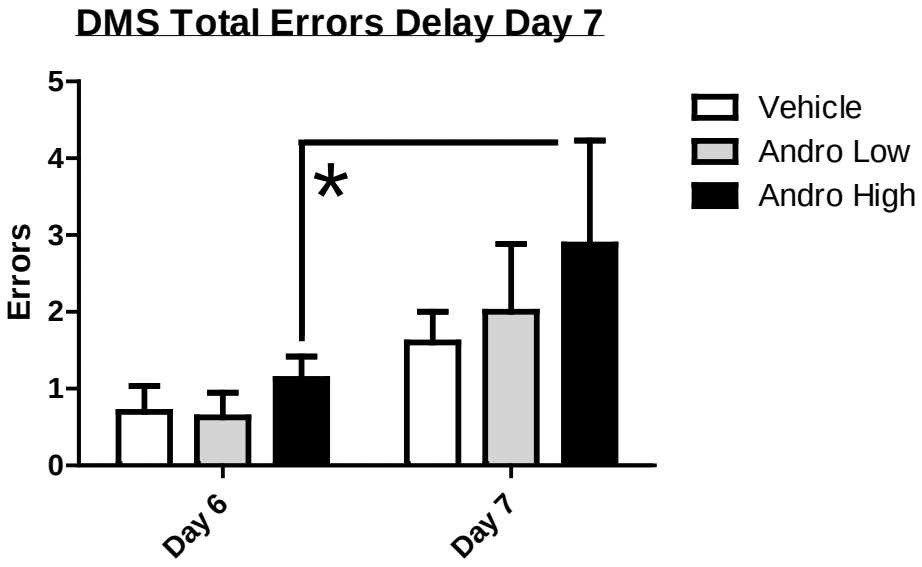

*Figure 6.* Mean (±SEM) swim distance (cm) during MM testing. (**a**) Swim distance across all days and all trials of testing, (**b**) Swim distance across days of testing. Androstenedione High group swam a significantly greater distance to find the platform across all three days and all six trials of testing compared to vehicle [$F(1,16) = 4.871$; $P = 0.042$]. There was also a significant linear trend with swim distance increasing as dose of androstenedione increases $F(1,2) = 182.905$; $P = 0.047$].

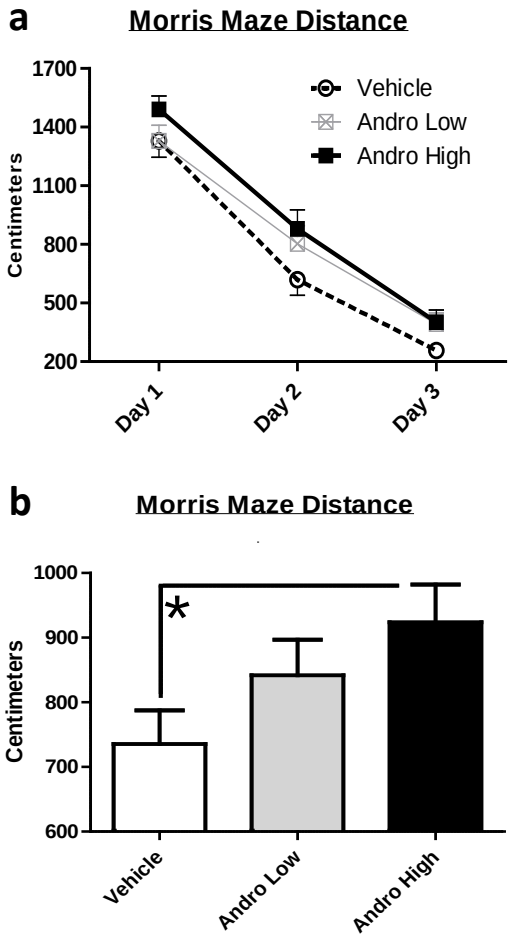

*Figure 7*. **a.** Mean (±SE) Total Open Arm Entries on the Elevated Plus Maze. There were no group differences. **b.** Mean (±SE) Total Time Spent in Open Arms (s) on the Elevated Plus Maze. There were no group differences.

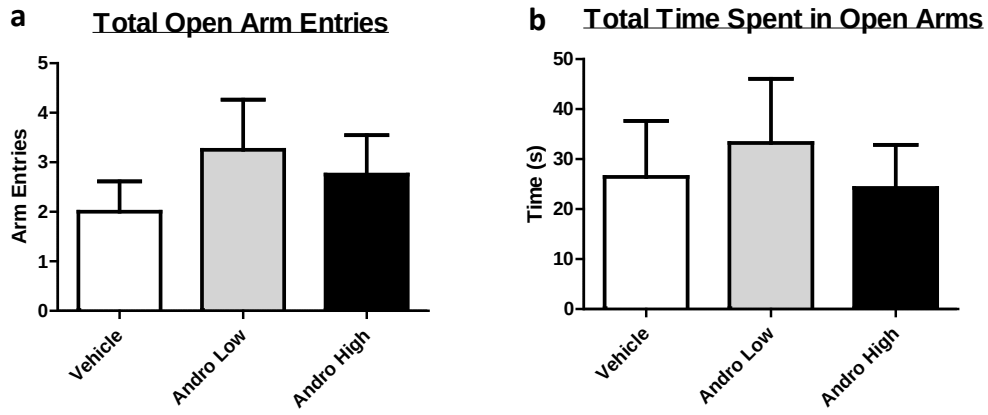

*Figure 8.* Mean (±SE) swim latency (s) on the Visible Platform. There were no group differences in swim latency.

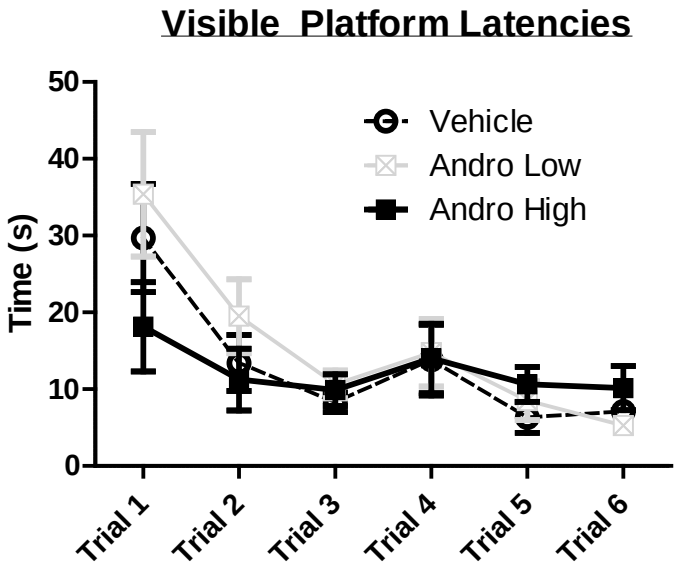

*Figure 9*. **a.** Mean (±SE) androstenedione serum levels (ng/ml). Both Androstenedione High [*t*(16) = 4.282; *P* = .0006] and Androstenedione Low [*t*(16) = 3.846; *P* = .0014] groups had higher androstenedione serum levels than the Vehicle group. No vehicle animal showed any detectable amounts of androstenedione. **b.** Correlation analysis indicated that higher androstenedione levels were associated with more WRAM working memory incorrect (WMI) errors on the last two days of testing when the working memory load was at its greatest (Trial 4) [r(25) = .609; *P* = .0007].

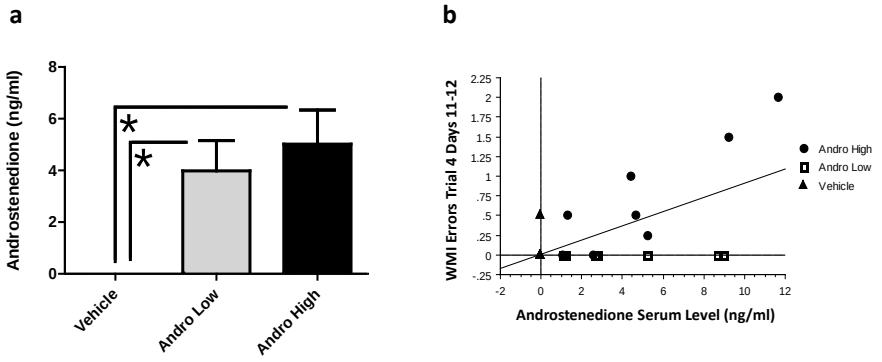

*Figure 10.* Correlation analysis indicated that higher GAD levels in the right entorhinal cortex were associated with swimming a greater distance to find the platform on the MM, collapsed across all days and all trials [r(23) = .431; *P* = .036] .

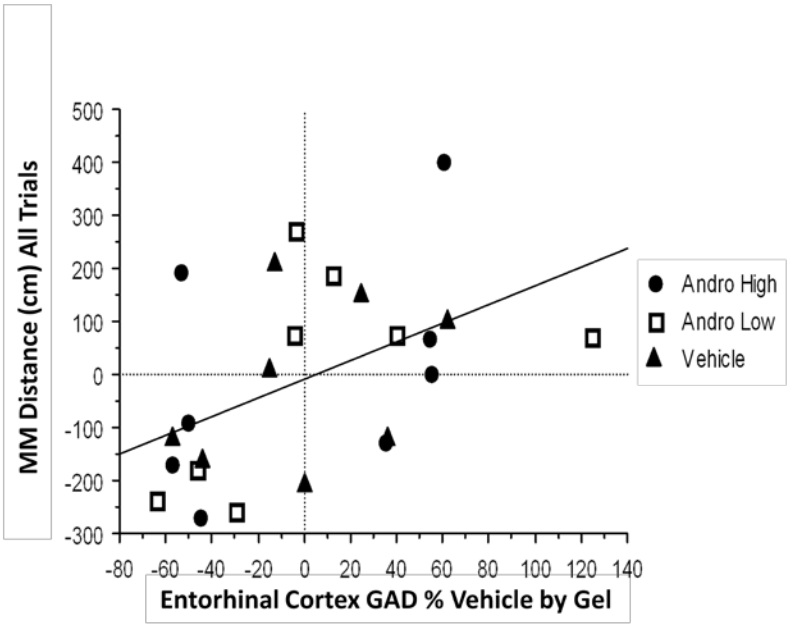

*Table 1.* Mean % Vehicle by gel (+SE) of GAD luminescence in the Frontal Cortex, Hippocampus, Entorhinal Cortex, Perirhinal Cortex, Cingulate Cortex, and Basal Forebrain. There were no group differences.

| | *Frontal Cortex* | *Cingulate Cortex* | *Basal Forebrain* | *Perirhinal Cortex* | *Entorhinal Cortex* | *Hippocampus* |
|---|---|---|---|---|---|---|
| *GAD Levels (% Control)* | | | | | | |
| *Vehicle* | *100.00 ± 6.39* | *100.00 ± 19.50* | *100.00 ± 21.61* | *100.00 ± 9.35* | *100.00 ± 14.26* | *100 ± 13.48* |
| *Andro Low* | *131.48 ± 27.28* | *130.27 ± 22.53* | *90.59 ± 23.21* | *145.00 ± 20.30* | *97.26 ± 20.86* | *93.54 ± 7.95* |
| *Andro High* | *123.23 ± 19.59* | *109.58 ± 21.54* | *99.11 ± 9.66* | *108.63 ± 18.22* | *92.47 ± 19.64* | *96.47 ± 15.05* |